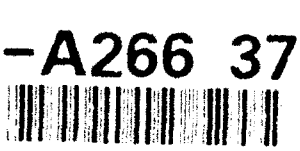

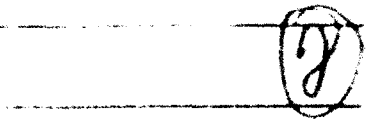

June 22, 1993 Final Report July 1, 1989 - Dec 31, 1993

Isolation of Thermostable Enzyme Mutants by Cloning and Selection in Thermophilic Bacteria

N00014-89-J-3134

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The objective of this project was to isolate thermostable variants of enzymes and to understand the molecular basis for their increased stability. Such variants may be isolated by introducing the gene encoding the enzyme into a thermophile and selecting for enzymatic activity at the high growth temperatures of these organisms. Thermostable mutants of kanamycin nucleotidyltransferase (KNTase) were obtained in this way and found to be also more resistant to chemical denaturants, to protease, and to inclusion body formation during their overexpression in Escherichia coli. The tools for obtaining mutants at up to 80°C by cloning in Thermus thermophilus, including a plasmid that transforms and expresses heterologous genes in this thermophile, were constructed, and more thermostable mutants of KNTase were isolated. Crystals of a thermostable KNTase were obtained and the structure was solved to 2.7A; comparison with the wildtype structure will allow correlation of the known genetic change with the structural basis for enhanced stability. Work was also begun on isolating thermostable mutants of another enzyme, chloramphenicol acetyltransferase.

Thermostability Thermophiles Kanamycin nucleotidyltransferase 4

Unclassified Unclassified Unclassified UL

UCLA School of Engineering and Applied Science

Attachment No. 1

to

EXPERIMENTAL STUDY OF NONASSOCIATED FLOW AND INSTABILITY OF FRICTIONAL MATERIALS

Poul V. Lade and Jerry A. Yamamuro

AFOSR Grant No. 91-0117

UCLA-ENG-93-26
APRIL 1993

INSTABILITY AND BEHAVIOR OF GRANULAR MATERIALS AT HIGH PRESSURES

JERRY A. YAMAMURO
POUL V. LADE

FINAL TECHNICAL REPORT

GRANT #: N00014-89-J-3134 R&T Code:4412070

PRINCIPAL INVESTIGATOR: Hans H. Liao

INSTITUTE: University of Wisconsin Biotechnology Center

GRANT TITLE: Isolation of Thermostable Enzyme Mutants by Cloning and Selection in Thermophilic Bacteria

AWARD PERIOD: JULY 1, 1989 - DECEMBER 31, 1992

OBJECTIVE:

Thermostable mutants of a test enzyme, kanamycin nucleotidyl-transferase (KNTase), were isolated previous to this granting period by cloning in the moderate thermophile *Bacillus stearothermophilus* and selection for the enzyme's activity at up to 70°C. The objectives of this research program were to elucidate the molecular basis for increased thermostability in these mutants and to extend this cloning and selection technology to *Thermus thermophilus*, capable of growth at >80°C.

EXPERIMENTAL APPROACHES:

1) To overexpress and purify wildtype and mutant KNTases to study their denaturation by agents other than heat and to solve their crystal structures.
2) To develop a system that expresses plasmid-borne heterologous genes in *Thermus thermophilus*.
3) To isolate more thermostable mutants of KNTase in *T. thermophilus*.
4) To isolate thermostable mutants of chloramphenicol acetyltransferase type III (CAT_{III}).

SUMMARY OF ACCOMPLISHMENTS:

Approach 1:

Overproduction of the KNTases was achieved by cloning the genes behind a phage T7 promoter and utilizing the T7 RNA polymerase system for heterologous gene expression in *Escherichia coli*. Overexpression of the wildtype KNTase led to the formation of inclusion bodies at 37° but not at 23°C, whereas the thermostable mutants were less susceptible to *in vivo* denaturation at 37°C. These results, indicating that the mutants have folding intermediates that are more stable in vivo, were documented in a publication (Liao, *Protein Expression and Purification* **2**: 43-50, 1991).

The KNTases that differ only in their thermostability (wild type, T130K, D80Y, and D80Y/T130K, in increasing order) form an ideal series with which to examine whether the stabilizing mutations protect only against heat denaturation or whether resistance to other denaturants is also enhanced in the mutants. Treatment of the purified KNTases by proteinase K, urea, Triton X-100, N-lauroylsarcosine, ethanol and tetrahydrofuran showed that in all cases the thermostable mutants retained more activity

than the wild type enzyme and displayed the same relative order as their resistance to heat denaturation (Liao, *Enzyme Microb. Technol.* **15**: 286-292, 1993).

Crystals of the D80Y/T130K variant were obtained (Kanikula, Liao, Sakon, Holden, and Rayment, *Arch. Biochem. Biophys.* **295**: 1-4, 1992) and its structure has been solved by J. Sakon, H. Holden and I. Rayment (UW Enzyme Institute) to 2.7Å, with an R value of <28%. The structure of the wild type (nonthermostable) KNTase will be the subject of further investigation by the Rayment-Holden group.

Characterization of the thermal denaturation of the KNTases by physical means (calorimetry and fluorescence spectroscopy) were not successful because this denaturation is irreversible.

Approach 2:

T. thermophilus was transformed using the system developed by Koyama (Koyama, Y., Hoshino, T., Tomizuka, N., and Furukawa, K., *J. Bacteriol.* **166:** 338-340, 1986). Selecting for complementation of a *trp*B mutation in the host strain by plasmid pYK109 (Koyama, Y., Arikawa, Y., and Furukawa, K., *FEMS Microbiol. Letts.* **72**: 97-102, 1990), the efficiency of transformation was $4x10^5$/μg with pYK109 isolated from *T. thermophilus* and $2x10^4$/μg with the plasmid prepared from *E. coli*. However, similar plasmids carrying the thermostable D80Y/T130K KNTase gene or a thermostable CAT gene did not yield any antibiotic-resistant transformants. Acting on the hypothesis that the KNTase gene did not have the appropriate signals for recognition by the *T. thermophilus* transcriptional machinery, a promoter probe vector was constructed in which DNA from a thermophile inserted upstream of the D80Y/T130K KNTase gene would drive expression of the antibiotic resistance marker. Such kan^R clones have been isolated but the DNA fragments that act as promoters in *T. thermophilus* have not been sequenced yet.

A similar construct was made by Y. Koyama and a promoter was isolated and sequenced. I modified his plasmid to generate a vector (pHT20) that will express heterologous genes in both *T. thermophilus* and *E. coli* and that will permit rapid sequencing of thermostable mutants. Thus all the tools necessary to clone and select for thermostable mutants in *T. thermophilus* are available.

Approach 3:

The D80Y/T130K KNTase gene was mutagenized by PCR-driven misincorporation and a mutagenized library was constructed in pHT20. Plasmid DNA from this library was transformed into *T. thermophilus* and kan^R clones were isolated at 74°C. These may carry KNTase mutants that are even more thermostable than those previously isolated in *B. stearothermophilus* and are currently being propagated and analyzed.

Approach 4:

CAT_{III} was chosen as another enzyme to stabilize because its crystal structure is known and its activity is readily selectable in eubacterial thermophiles. Conditions for the selection of thermostable mutants of

CAT_{III} in *B. stearothermophilus* were identified, and vectors for *in vitro* mutagenesis of this gene were made. However, even though a library of mutagenized CAT_{III} genes was made, this project was not completed. In the course of constructing vectors for this approach, we found that the D80Y/T130K KNTase was a significantly better transformation marker for *B. stearothermophilus* than the wildtype gene even at 47°C (Liao and Kanikula, *Current Microbiology* **21**: 301-306, 1990).

INDEX OF PUBLICATIONS:

Liao, H.H. and Kanikula, A.M. (1990) "Increased efficiency of transformation of *Bacillus stearothermophilus* by a plasmid carrying a thermostable kanamycin resistance marker." *Current Microbiology* **21**: 301-306.

Liao, H.H. (1991) "Effect of temperature on the expression of wild-type and thermostable mutants of kanamycin nucleotidyltransferase in *Escherichia coli*." *Protein Expression and Purification* **2**: 43-50.

Kanikula, A.M., Liao, H.H., Sakon, J., Holden, H.M., and Rayment, I. (1992) "Crystallization and Preliminary Crystallographic Analysis of a Thermostable Mutant of Kanamycin Nucleotidyltransferase." *Arch. Biochem. Biophys.* **295**: 1-4.

Liao, H.H. (1993) "Thermostable mutants of kanamycin nucleotidyltransferase are also more stable to proteinase K, urea, detergents, and water-miscible organic solvents." *Enzyme Microb. Technol.* **15**: 286-292.

No patents were filed.